

**Title: Multispecies diel transcriptional oscillations in open ocean
heterotrophic bacterial assemblages**

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CONFIDENTIAL: Accepted, Science magazine, 5/22/2014:

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Abstract: Oscillating diurnal rhythms of gene transcription, metabolic activity and behavior are found in all three domains of life. Diel cycles in naturally occurring heterotrophic bacteria and archaea however, have rarely been observed. Here we report time-resolved whole genome transcriptome profiles of multiple, naturally occurring oceanic bacterial populations sampled *in situ* over three days. As anticipated, the cyanobacterial transcriptome exhibited pronounced diel periodicity. Unexpectedly however, several different heterotrophic bacterioplankton groups also displayed diel cycling in many of their gene transcripts. Furthermore, diel oscillations in different heterotrophic bacterial groups suggested population-specific timing of peak transcript expression in a variety of metabolic gene suites. These staggered multispecies waves of diel gene transcription may influence both the tempo and mode of matter and energy transformation in the sea.

Main Text:

The coordination of biological activities into daily periodic cycles is a common feature of eukaryotes and is widespread among plants, fungi, and animals, including man (1). Among single celled non-eukaryotic microbes, diel cycles have been well documented in cyanobacterial isolates (2-4), one halophilic archaeon (5), and in bacterial symbionts of fish and squid (6, 7). Some evidence for diel cycling in microbial plankton has also been suggested on the basis of bulk community amino acid incorporation, viral production, or metabolite consumption (8-10). The existence of regular diel oscillations in free-living heterotrophic bacterial species however, has rarely been assessed.

Microbial community RNA sequencing techniques now allow simultaneous determination of whole genome transcriptome profiles among multiple co-occurring species (11, 12), enabling high frequency, time resolved analyses of microbial community dynamics (12, 13). To better understand temporal transcriptional dynamics in oligotrophic bacterioplankton communities, we conducted a high-resolution multi-day time series of bacterioplankton sampled from the North Pacific Subtropical Gyre (14).

To facilitate repeated sampling of the same planktonic microbial populations through time, automated Lagrangian sampling of bacterioplankton was performed every two hours over three days using a free-drifting robotic Environmental Sample Processor (ESP; (13, 15); Fig. S1). Following instrument recovery, planktonic microbial RNA was extracted, purified and converted to cDNA to assess whole genome transcriptome dynamics of predominant planktonic microbial populations (Table S1, Table S2). The recovered cDNAs were dominated by transcripts from *Prochlorococcus* and several proteorhodopsin-containing or photoheterotrophic bacteria,

including members of the *Pelagibacter* (SAR11), *Roseobacter*, SAR116, SAR86, and SAR324 clades (Fig. S2).

Phylogenetic analysis of gene transcripts in the most abundant taxa revealed the presence of some microdiversity (Figs. S3-8). The most abundant transcripts sampled at any given time point however, were dominated by only a few genotypes within each population that persisted throughout the sampling period. An exception was *Roseobacter*, with transcripts for two different genes (*groEL* and *dnaK*) indicating the presence of a genotype that started at a very low abundance and increased in representation over the course of the time series. This variability could be due to an injection of a new population as water masses mixed during the latter portion of the time series, or possibly to an alteration in the relative transcriptional activities of two ecotypes that are responding to changes in the surrounding environment.

Transcriptional activity in *Prochlorococcus* was highly dependent on the time of day. Harmonic regression analyses indicated that nearly half (1,491) of all *Prochlorococcus* population transcripts were significantly periodic (Table 1; Table S3; Fig 1). The expression patterns observed were similar to those of monocultures growing in controlled laboratory settings (4) but there were also notable differences (Fig. 1). For example, photosystem I gene expression exhibited a double peak in the wild *Prochlorococcus* transcriptome around noon (Fig. 1). In contrast, under laboratory conditions most photosystem I genes, i.e. *psaL* and *psaF*, were found to peak just before noon, while *psaA* and *psaB* peaked shortly after noon (4).

The largest discrepancy between *Prochlorococcus* laboratory studies and our field observations was that a considerable number of *Prochlorococcus* transcripts in our field populations peaked around midday (Fig. 1). Some of these genes did exhibit periodicity in cultures, but peaked at a different time of day in field populations. A larger fraction of these

mid-day peaking transcripts were either not periodically expressed, or were not present in the culture experiments. In addition, 62% of the 10am - 4pm peaking transcripts in our field study lacked KEGG orthology annotations, as opposed to those peaking in the evening or late at night.

A number of factors may be responsible for differences in transcript dynamics between in laboratory cultures versus field *Prochlorococcus* populations. Maximal light levels at our study site at 23 m depth were frequently two fold higher ($450 \text{ } \mu\text{mol Q/m}^2/\text{s}^{-1}$) than those used in laboratory microarray experiments ($232 \text{ } \mu\text{mol Q/m}^2/\text{s}^{-1}$) (4). Fundamental genetic differences between our field populations and the *Prochlorococcus* strain used in laboratory culture experiments likely also contribute to the differences we observed. Other variables, including nutrient composition and organismal interactions, may also be a factor in the observed differences. While we could not identify obvious trends in the type or function of transcripts showing peak expression during the mid-day period, they did include a wide range of enzymatic functions that are more consistent with nutrient-responsive metabolic changes rather than a simple high-light stress response.

An abundant *Roseobacter* population also showed strong diel oscillations in its transcriptome profile, most notably in expressed genes involved in bacteriochlorophyll-associated aerobic anoxygenic photosynthesis (AAnP). Overall, a large fraction of *Roseobacter* transcripts were periodically expressed (Table 1). Of these, the majority peaked during daylight hours, with only a few gene transcripts peaking at night (Fig. 2). While this pattern contrasts with that observed in *Prochlorococcus*, where most diurnally regulated transcripts peaked at dawn or dusk, it was consistent with transcriptional regulation recently reported in *Dinoroseobacter shibae* (16).

Thirty five of the forty significantly periodic *Roseobacter* transcripts that peaked between 11 pm and 7 am encoded genes belonging to a large photosynthetic “superoperon” (Fig. S9). Nightly expression of these genes, followed by immediate repression upon light onset, is consistent with the *D. shibae* study (16), and may be preparing cells for efficient solar energy harvest in the early morning hours. Functions that peaked during the day-time hours included ribosomal proteins, respiratory transcripts, genes involved in amino acid metabolism, and transporters (Fig. 2).

Proteorhodopsin-containing photoheterotrophs including members of the SAR11, SAR116, and SAR86 also showed evidence of diel periodicity in many of their gene transcripts (Fig. 2, Table 1). Interestingly, all opsin-containing bacteria analyzed (SAR11, SAR116, SAR86, and SAR324) exhibited statistically significant diel oscillations in their proteorhodopsin gene transcripts (Table S3; Fig. S10). Peak expression of the opsin transcripts occurred near dawn in all these populations (Fig. S10), potentially optimizing solar energy capture by the light-driven, proton-pumping rhodopsins.

Principal components analysis distinguished time series samples for each heterotroph by time of day (Fig. 3) and showed significant correlation with the light-driven behavior of *Prochlorococcus* (Table 1). Overall, this data is consistent with profound, genome-wide transcriptional changes across the day-night cycle for each population. In addition, co-clustering of transcripts using GeneARMA (GA; (14, 17)) revealed suites of gene transcripts that exhibited similar expression patterns among different taxa (Fig. 4; Fig. S11-14; Table S4). For example, a group of transcripts that fit highly similar GA expression models across multiple species included Pro GA5, Pro GA7, Pro GA9, Pro GA23, SAR11 GA6, SAR11 GA18, SAR116 GA2, and *Roseobacter* GA8 (Fig 4; Fig S14; Table S4). These multispecies, day-peaking transcripts

(Fig S14; Table S4) included gene products associated with respiration (*Prochlorococcus*, SAR11, SAR116, *Roseobacter*), nitrogen metabolism (*Prochlorococcus*, SAR11, SAR116), glycine metabolism (*Prochlorococcus*, SAR11, *Roseobacter*), carbon monoxide metabolism (SAR116, *Roseobacter*) and DNA synthesis (*Prochlorococcus*, *Roseobacter*). This co-clustering of gene transcripts reveals a complex pattern of expression through the day and across the time series, and provides evidence for parallel trends in gene expression across multiple species (Fig. 4, Fig. S11-14, Table S4).

Together, the transcriptional profiles of *Roseobacter*, SAR11, SAR116 and SAR86 indicate diel cycling of metabolic gene transcripts, and suggest a multispecies wave-like progression of upregulated gene suites across the day/night cycle (Fig. 4). Most conspicuously, a regular diel succession of translational, transcriptional and respiratory gene transcripts was followed by peaks in transporter transcripts that possibly reflect a metabolic recovery phase (Fig. 2). Many of these metabolic pathway transcripts peaked earlier in the day in *Roseobacter* field populations relative to other bacterial heterotrophs (Fig. 2, Fig 4, Table S4).

The overall transcriptional profile of SAR324 did not show as many transcript diel oscillations as other heterotrophic taxa. Instead, principal components analysis clustered SAR324 transcripts according to the day that they were collected (Fig. 3). In particular, the SAR324 group showed a strong separation between the first portion of the time series and the second in principal components analysis (Fig. 3). This split appears to be associated with the increases in temperature and salinity observed across the time series (Fig. S1).

The diurnal patterns reported here for open ocean heterotrophic bacterioplankton were different from those observed in a previous study of phylogenetically related coastal bacterioplankton using similar methods (12). For example, coastal versus open ocean SAR11

populations revealed differential expression levels among several orthologous transcript categories (Fig. S15). Additionally, while the open ocean SAR11 populations reported here exhibited statistically significant diel oscillations for many gene transcripts (Fig. 2), the coastal SAR11 populations did not.

Currently available data are insufficient to provide definitive mechanistic explanations for the diel behaviors we observed in different heterotrophic bacterioplankton species. It is possible that photoreceptors in these bacteria are involved in regulating light-dark cycles of transcriptional activity. Marine *Roseobacter* species have previously been shown to regulate their global transcriptional behavior in response to light (16), and laboratory cultures of *Pelagibacter* also exhibit light-responsive metabolic behaviors (18). Differences between the behaviors of SAR11 coastal versus open ocean field populations however (Fig. S15), as well as comparisons of several taxa in our field study versus laboratory experiments on related cultivated isolates (Fig. 1), suggest that other factors may be at play in regulating diel behavior among these different bacterioplankton populations.

Previous studies have proposed that tight metabolic coupling between primary producers and consumers in microbial plankton might elicit conspicuous diel cycling in heterotrophic bacterial activities (8). The diel cycling we observed among different bacterioplankton species is consistent with this hypothesis, with multiple co-existing heterotroph populations exhibiting diurnal oscillations resembling those of their photoautotrophic neighbors. We postulate that the tightly coupled multispecies temporal expression patterns observed may elicit corresponding waves of species-specific metabolic responses at regular time intervals, potentially coordinating diverse biogeochemical activities in these complex microbial communities. Such temporal coordination of biogeochemical activities among multiple species may be important regulators of

both the tempo and mode of microbial matter and energy transformation in the sea.

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Acknowledgments:

We thank the officers and crew of the Kilo Moana, Chief Scientist Julie Robidart, Sam Wilson, and the ESP engineering and Science team (John Ryan, Jim Birch, Chris Preston, Gene Massion, Scott Jensen, Brent Roman) for all the able assistance. This work was supported by grants from the Gordon and Betty Moore Foundation GBMF #492.01 and #3777 (EFD) and NSF grant EF0424599 (EFD). Development of the ESP was supported by National Science Foundation Grant OCE-0314222 (to C.A.S.), National Aeronautics and Space Administration Astrobiology Grants NNG06GB34G and NNX09AB78G (to C.A.S.), the Gordon and Betty Moore Foundation (C.A.S.), and the David and Lucile Packard Foundation. This work is a contribution of the Center for Microbial Oceanography: Research and Education (C-MORE). Sequences reported in

259 this paper have been deposited in the Gen-Bank database (accession no. SRP041215).

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Supplementary Materials:

Materials and Methods

Figures S1-S14

Table S1

Legends for External Tables S2-S4

References (27-43)

Fig. 1. Laboratory versus field comparisons of periodic expression patterns in *Prochlorococcus* populations. **A.** Scatter plot shows time of peak abundance for 973 transcripts identified as significantly periodic in both studies. Histograms show the total number of genes peaking in 1-hour intervals in this study (top) and the laboratory experiment (side). Black bars represent genes identified as significantly periodic in both studies, grey bars represent genes expressed in both studies but significantly periodic in only one, and white bars represent significantly periodic transcripts that were not detected in the other dataset. For this comparison, we used published significance cutoffs from the laboratory study (4), but for consistency generated new peak times using our harmonic regression approach and the published normalized mean expression levels for each time point. In general, the peak times generated using our approach closely matched published values for that dataset. **B-C.** Plots showing relative expression (normalized to mean expression level) over time for our metatranscriptome (top trace) and in microarray data (bottom trace) for selected transcripts. For comparison, experimental midnights (24 hr and 48hr) from the microarray study are aligned with the 12:00AM samples from 9/9 and 9/10, respectively. All ATP synthase subunits (**B**) and selected subunits from photosystem I (**C**) are shown.

Fig. 2. Timing of periodically expressed transcripts. For each population, a histogram showing the number of periodically expressed diel transcripts with peak expression within 1-hr intervals throughout the day is shown (left). On the right, time of peak expression of all transcripts assigned to selected KEGG pathways is plotted (grey). Red circles denote transcripts identified as significantly periodic (24 hour period). The “transporters” category includes both the ABC Transporters KEGG pathway and the Transporters BRITE hierarchy, the “photosynthesis” category includes both the Photosynthesis KEGG pathway and the BRITE Photosynthesis Proteins categorizations. “Carbon Fixation” refers to genes assigned to the Carbon Fixation in Photosynthetic Organisms KEGG Pathway. The photosynthesis and carbon fixation categories are present in heterotrophic organisms due to cross-assignment of ATP Synthase genes and pentose phosphate cycle genes. Black and yellow bars depict the daily photoperiod (based on sunrise and sunset times).

Fig. 3. Principal Component analysis of population transcriptional profiles. Transcript abundances were normalized to total transcripts assigned to each population at each time point,

and arcsin transformed to approximate normality (19). Symbol color denotes time of day and shape denotes day of collection. Grey lines connect samples to centroids for selected sample groupings that separate points well. *Roseobacter* SAGs: samples collected between 7am and 9pm (vs. 9pm to 7am); SAR11, SAR116 and SAR86 cluster, samples collected between 9am and 6pm (and vice versa); SAR324 cluster, samples collected before or after 9/9 4pm. All factor correlations shown were highly significant ($p = 0.001$). Alternative time of day categories were also highly significant for *Roseobacter* SAGs, SAR11, SAR116 and SAR86. SAR116 (r^2 0.10, p 0.037) and SAR86 (r^2 0.12, p 0.019) also correlated weakly with the grouping shown for SAR324. All analyses carried out using functions in the vegan software package (20).

Fig. 4. Timing of expression of functional gene clusters in different taxa clustered by similarity. Heatmap shows cluster models for all geneARMA clusters, colored by mean-centered relative expression (red=high, blue=low). Black and yellow bars show the daily photoperiod. Each box represents a single sampling event, for sample times see Table S1. Dendrograms show cluster model similarity (Pearson correlations, average linkage clustering, scale bar at upper right represents a correlation of 0.5). The total number of genes (A), significantly periodic genes (B), and genes associated with Photosynthesis (C), Ribosome (D), Oxidative Phosphorylation (E), Amino Acid Metabolism (F), and Transport (G) (defined as for Figure 2), are listed for each cluster.

Table 1: Harmonic Regression Results

	Prochlorococcus	Roseobacter SAGs	SAR11	SAR116	SAR86	SAR324
Sequence reads ¹	2886677	177982	774064	200368	151468	118098
Transcripts ²	3045	2604	2802	2618	2367	4732
Periodic ³	1491	426	201	80	10	8
Constrained PCA vs 24-hour clock ⁴	0.68 (p = 0.005)	0.49 (p = 0.005)	0.24 (p = 0.005)	0.15 (p = 0.005)	0.13 (p = 0.005)	0.10 (p = 0.01)
Procrustes Test vs. Prochlorococcus PCA ⁵		0.78 (p < 0.001)	0.55 (p < 0.001)	0.70 (p < 0.001)	0.52 (p < 0.001)	0.36 (p = 0.031)
Mantel Test vs. Prochlorococcus ⁶		0.63 (p < 0.001)	0.40 (p < 0.001)	0.31 (p = 0.003)	0.26 (p = 0.005)	0.27 (p = 0.002)

¹ The total number of sequence reads assigned to each taxon bin

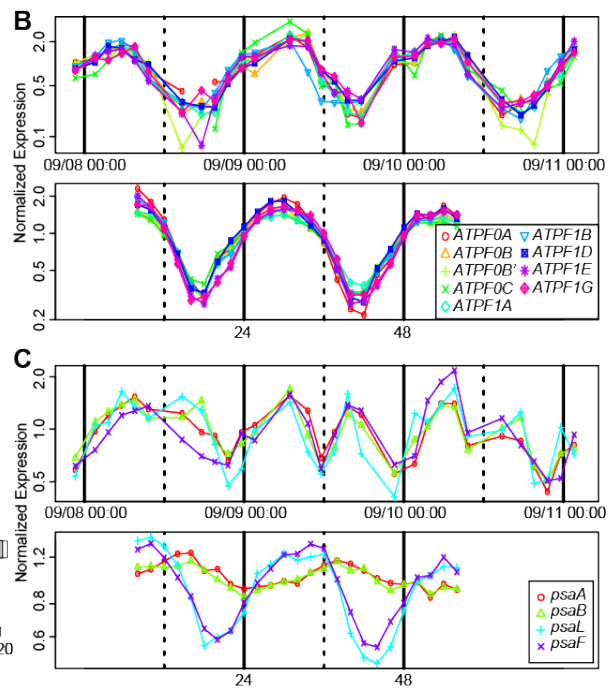
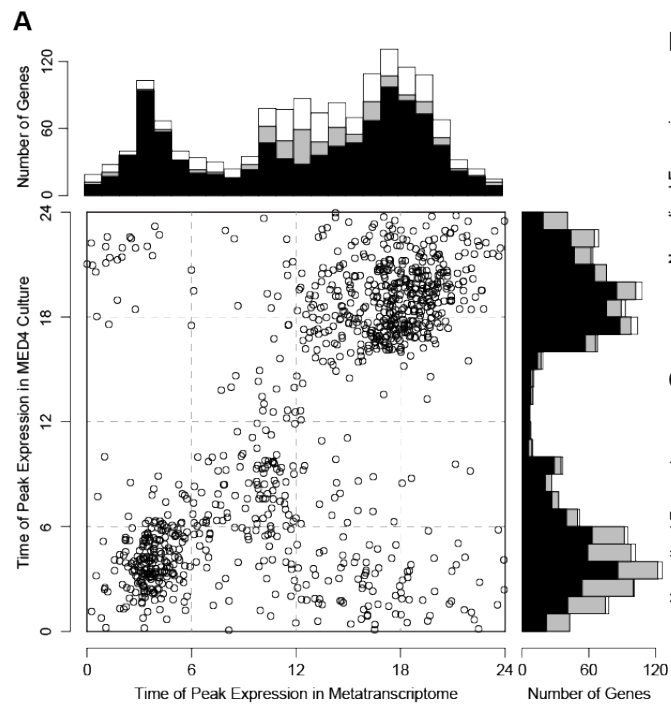
² The total number of unique ortholog clusters (see Database S1) with at least one mapped sequence.

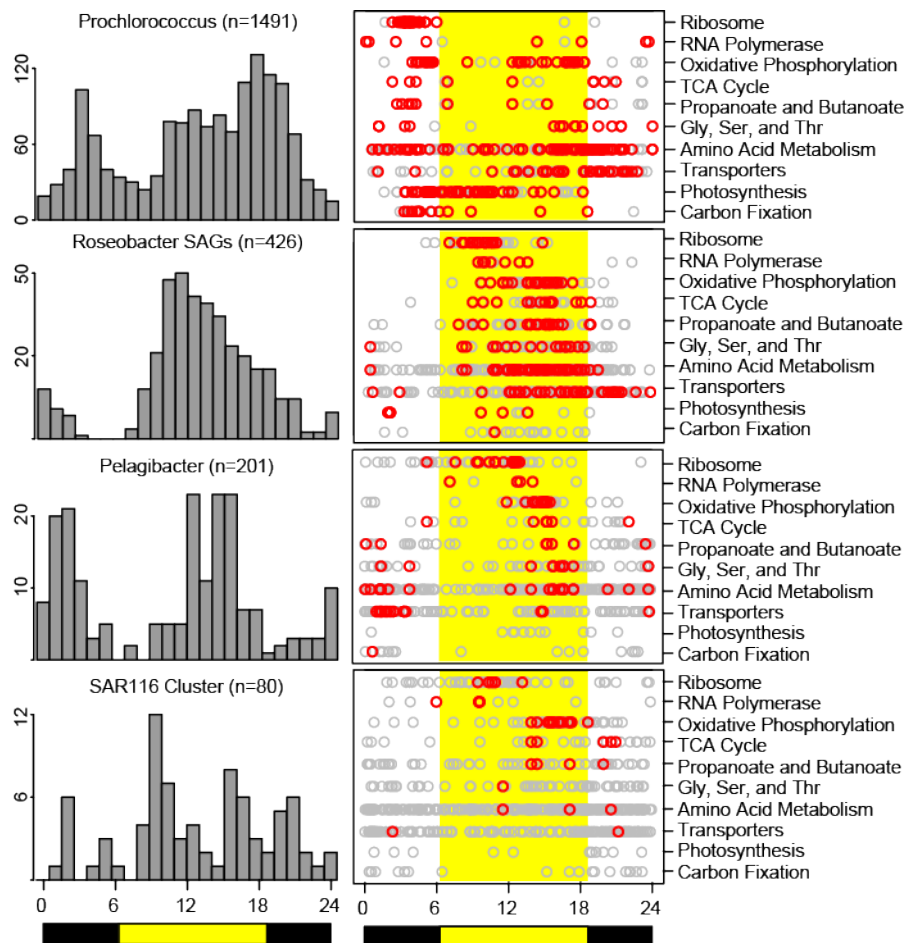
³ The total number of sequences identified as showing 24-hour periodicity using harmonic regression.

⁴ Proportion of variance explained by 24-hour periodicity in constrained principal components analysis.

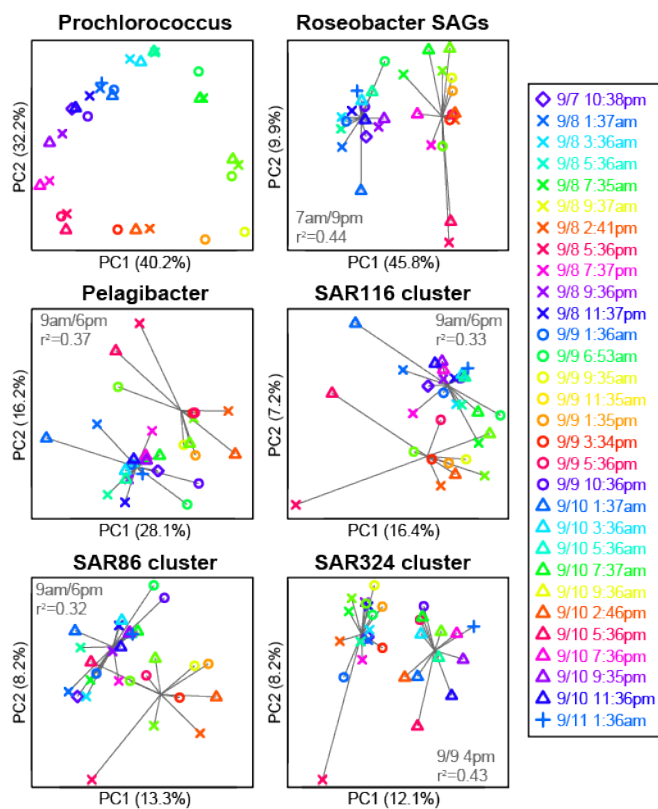
⁵ Procrustes correlation between the first two principal components from *Prochlorococcus* and other taxa (unconstrained principal components analysis as shown in Fig. 3). P-value based on 999 permutations.

⁶ Correlation between pairwise sample similarities from *Prochlorococcus* and heterotrophic taxa based on Mantel test on Euclidean distance matrices. P-value based on 999 permutations.





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